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Tetracycline resistance and genotypes of *Streptococcus zooepidemicus* isolated from Thoroughbred horses with respiratory disease in Japan

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Tetracycline resistance has become widespread in *Streptococcus zooepidemicus* in horses in many countries, and tetracycline-resistant *S. zooepidemicus* is being isolated from respiratory specimens in Japan. *Streptococcus zooepidemicus* is reportedly not a homogeneous clonal population but consists of a wide diversity of strain types. Here, we examined the relationship between tetracycline resistance determinants and *S. zooepidemicus* genotypes. We used 66 isolates of *S. zooepidemicus* derived from Thoroughbred horses in Japan that had developed signs of respiratory disease between 2000 and 2014. Minimum inhibitory concentrations (MICs) of tetracycline and minocycline for the isolates were measured, and eight PCR methods, detecting the tetracycline resistance determinants tetO, tetW, tetM, tetL, tet40, tetK, tetQ, and tetT, were applied to the 66 isolates. Twenty (30.3%) of the *S. zooepidemicus* isolates were tetracycline resistant, and 11 (16.7%) were minocycline resistant (Table 1). In 2010–2014, 50% (9/18) of *S. zooepidemicus* were tetracycline resistant; moreover, isolation rates of *S. zooepidemicus* with minocycline resistance were significantly greater than in 2000–2009. All tetracycline-resistant *S. zooepidemicus* possessed one of the tetracycline resistance determinants; 12 isolates had tetO, 6 had tetW, and 2 had tetM. The MICs of minocycline for *S. zooepidemicus* possessing tetO were significantly higher than those for *S. zooepidemicus* with tetW; interestingly, *S. zooepidemicus* with tetO were isolated more frequently in 2010–2014 than in 2000–2009. Two PCR typing methods based on characterization of the M-protein hypervariable (HV) region and the 16S-23S rRNA gene intergenic spacer were applied to the 66 isolates to determine *S. zooepidemicus* genotypes. As in other studies, *S. zooepidemicus* represented a wide diversity of strain types: the 66 isolates of *S. zooepidemicus* comprised 15 different types of varying prevalence. The three most common types, A1HV4, A1HV5, and C1HV2, accounted for 50% of all the typed isolates. Although the *S. zooepidemicus* isolates belonging to certain genotypes (e.g. A1HV2, A1HV3, and D1HV5) did not possess any of the tetracycline resistance determinants, all five A1HV1 strains possessed tetO, all two B2D2HV3 had tetW, and the single D2HV5 strain had tetO. It is possible that these strains acquired their resistance genes before their introduction into Japan's Thoroughbred population. Finally, we compared two phylogenic trees based on PCR – restriction fragment length polymorphism (PCR-RFLP) analysis and the tetO sequences of 12 *S. zooepidemicus* isolates. The same distributional differences among each isolates were observed in the two types of phylogenic tree. The results indicated that the tetO gene of *S. zooepidemicus* might not have been transferred by plasmid or conjugative transposon. These results should help us to understand the relationship between tetracycline resistance determinants and *S. zooepidemicus* genotypes and to select antimicrobials for horses with respiratory tract infections.

Table 1
Isolation rates of *S. zooepidemicus* with tetracycline or minocycline resistance

	Rates of resistant strain[a]			
Antimicrobials	2000-2004 (n=30)	2005-2009 (n=18)	2010-2014 (n=18)	Total (n=66)
Tetracycline	23.3%	22.2%	50.0%	30.3%
Minocycline	10.0%[†]	5.6 %[†]	38.9%	16.7%

a) Strains with MICs of greater than or equal to 8μg/ml were considered to be resistant.
[†] Significantly smaller than in 210-2014 ($P < 0.05$, Fisher's exact test)

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Phospholipase A_2 of *Streptococcus equi*: an ambiguous virulence factor.

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Phospholipase A_2 (PLA_2) toxins are enzymes that hydrolyse phospholipids and are likely to be involved in the disruption of eukaryotic cell membranes and host invasion[1]. The production of PLA_2 toxin, SlaA, by *Streptococcus pyogenes* was associated with increased morbidity and mortality in humans[2]. *Streptococcus equi* subspecies *equi* (*S. equi*) is the causal agent of strangles, an infectious disease of horses characterised by abscessation of the lymph nodes of the head and neck[3]. SlaA from *S. pyogenes* M3 and *S. equi* strain 4047 (*Se*4047) share 98% predicted amino acid sequence identity[4]. *Se*4047 also encodes a second putative PLA_2 toxin, SlaB. The role and activity of SlaA and SlaB from *S. equi in-vivo* have not been investigated yet. This work aims to confirm the production of SlaA in horses during *S. equi* infection and whether its presence increases the pathogenicity of *S. equi* strains *in-vivo*. The production of an SlaA-specific antibody was measured by ELISA using serum collected from horses suffering from strangles. The importance of SlaA and SlaB role *in-vivo* was determined by experimental infection of Welsh mountain ponies with either a wild type *S. equi* strain (WT-*Se*) or a strain lacking genes encoding PLA_2 (Δ*slaAB*-strain). The level of antibodies against recombinant SlaA was significantly higher in sera from horses recently exposed to *S. equi* whilst the results were lower in sera from healthy horses. This result supports SlaA production and immunogenicity *in-vivo*. However, no significant attenuation of the disease was observed after infection with the Δ*slaAB*-strain when compared with ponies infected with WT-*Se*. Nevertheless, macroscopic post-mortem examination identified that the abscesses in ponies infected with the Δ*slaAB*-strain were smaller and appeared to be confined to individual nodules of the lymph node. On the other hand, those abscesses caused by the WT-*Se*, were larger and diffuse. Regarding cytokine production, differences in the levels of TNFα were observed during the course of the infection in ponies challenged with the *ΔslaAB*-strain when compare to those infected with WT-*Se*, suggesting that PLA_2 toxins exert a regulatory effect on the inflammatory response. In conclusion, SlaA and SlaB are potential virulence factors produced by *S. equi* during infection. Post-mortem observations indicated that the production of SlaA and SlaB may increase the size of lymph node abscesses *in-vivo*. SlaA and SlaB also regulate the expression of TNFα during the course of infection. Alongside preliminary *in-vitro* results obtained by our group, these results suggest that PLA_2 toxins play a subtle role in the pathogenicity of *S. equi* that may involve the innate immune response.

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